SUBSTITUTE SPECIFICATION
Clean Version

DESCRIPTION

METHOD OF INHIBITING BONE RESORPTION BY FOR INHIBITING OSTEOCLAST FORMATION

Technical Field

The present invention relates to a method for inhibiting osteoclast formation , or a method for inhibiting bone resorption.

More particularly this invention relates to a method for inhibiting osteoclast formation or a method for inhibiting bone resorption by ultrasound (which may be called as "US" hereinafter) treatment.

Background Art

A known and conventional method for inhibiting osteoclast formation or for inhibiting bone resorption includes a method consisting of adding a bisphosphonate to a co-culture system [Bilezikian, JP., Raisz, LG., and Rodan, GA. eds., "Principles of bone biology," Academic Press, New York (1996)]. Clinically, a medicine effective in inhibiting osteoclast formation or inhibiting bone resorption has been used for treating of a bone disease caused by abnormal bone resorption due to osteoclast [Bilezikian, JP., Raisz, LG., and Rodan, GA. eds., "Principles of bone biology," Academic Press, New York (1996)].

In the method for adding or administering of a medicine or pharmaceuticals, however, the medicine or pharmaceuticals are taken into body or cells directly, and thus it is not free from the risk of causing some side-effects. Therefore, there is a demand for a method whereby it is possible to inhibit osteoclast formation or bone resorption, while being less likely to cause side-effect than does the conventional method.

In view of this, the present inventors had tried hard to find such a method as mentioned above, that is, a method whereby it is possible to inhibit osteoclast formation or bone resorption, in spite of being less likely to cause side-effect than does the conventional method, and had observed that stimulation with ultrasound, which is physical and non invasive, can

effectively inhibit osteoclast formation or bone resorption, and achieved this invention.

Disclosure of the Invention

The present invention provides a method for inhibiting osteoclast formation or a method for inhibiting bone resorption which comprises exposing cells to ultrasound in the culture containing an osteoclast precursor and an inducing factor of osteoclast formation and/or bone resorption, or a method for inhibiting osteoclast formation or a method for inhibiting bone resorption which comprises exposing cells to ultrasound in the co-culture containing an osteoclasst precursor and a supporting cell for osteoclastogenesis.

Brief Description of the Drawings

Figure 1 shows the number of osteoclasts over time in an experiment where the development of mouse osteoclast precursors into osteoclasts is stimulated by sRANKL.

Figure 2 shows the number of osteoclasts obtained when mouse osteoclast precursors have been co-cultivated with mouse bone marrow cells for 96 hours.

Figure 3 shows the number of osteoclasts obtained when mouse osteoclast precursors have been co-cultivated with mouse bone marrow cells for 120 hours.

Figure 4 shows the area of resorption pits by osteoclasts which have been derived from mouse oseteoclast precursors co-cultivated with mouse bone marrow cells.

Note that, for all the relevant Figures, * represents the test result is significantly different from the result of control group at $p<0.05$ based on the Student's t-test, while *** represents the same at $p<0.001$.

Best Mode for Carrying Out the Invention

The present invention is described below in detail.

The osteoclast precursor used in this invention is the precursor that

develop into osteoclasts in the presence of an inducing factor added to the culture medium, or in the presence of supporting cells added to the culture medium which have been known from the effect to other osteoclast precursors to have an activity to support osteoclast formation. As such osteoclast precursors, bone marrow-derived precursors, spleen-derived precursors and blood monocyte-derived precursors may be mentioned. Those precursor cells may be obtained from the bone marrow, spleen or peripheral blood of humans, mice, etc., and they can be cultivated through passage on a culture medium specially prepared for the emphasized growth of such osteoclast precursors.

The inducing factor which stimulates osteoclast precursors to turn them into osteoclasts is at least one chosen from the group comprising a macrophage colony stimulating factor (to be referred to as "M-CSF" hereinafter), osteoclast forming factor (RANKL/TRANCE/ODF/OPGL, or to be referred to as "RANKL" hereinafter), tumor necrosis factor (the same as above "TNF"), interleukin 4 (IL-4), and vascular endothelial cell growth factor (VEGF). Those factors may be used alone or in combination. RANKL is preferably used, or an artificially synthesized soluble RANKL is most preferred when used at a concentration of 1 to 250 ng/ml. Addition of the inducing factor into the culture medium should be occurred during a period immediately after the inoculation of osteoclast precursors onto the medium till osteoclast formation .

Besides the osteoclast precursors, the co-culture system may include at least one chosen from the group comprising osteoblasts, stroma cells, fibroblasts, T-cells and B-cells. A single kind or several kinds of those cells may be included.

Moreover, the co-culture system preferably includes an inducing factor. The inducing factor may include, in addition to M-CSF, RANKL, TNF, IL-4 and VEGF mentioned above, interleukin-1 (to be referred to as "IL-1" hereinafter), interleukin-3 (the same as above "IL-3"), interleukin-6 (the same as above "IL-6"), interleukin-11 (the same as above "IL-11"), interleukin-15 (the same as above "IL-15"), interleukin-17 (the same as above "IL-17"), prostaglandins (the same as above "PG") including prostaglandin E2,

parathyroid hormone (the same as above "PTH"), parathyroid hormone-related peptide (the same as above "PTHrP"), granulocyte/macrophage colony stimulating factor (the same as above "GM-CSF"), and activated vitamin Ds including 1 α,25-dihydroxyvitamin D3 (the same as above "VD"). They may be used alone or in combination.

Of all theinducing factors cited above, RANKL is preferred to be used. This is because RANKL is a factor essential for the osteoclast formation, and plays an important role in bone metabolism/maintenance [Takahashi, N., et al., Biochem Biophys Res Comm 256: 449-455 (1999)]. RANKL is mainly present on the surface of cells known to support the osteoclast formation, such as osteoblasts, stroma cells, fibroblasts, etc., and induces osteoclast formation by binding with receptors (RANK/TRANCER) of the osteoclast precursors [Takahashi, N., et al., Biochem Biophys Res Comm 256: 449-455 (1999)]. Because RANKL hardly exist as a soluble factor in the body, the co-culture system for the osteoclast formation may be thought as a system resembling an in-situ environment involved in the bone metabolism. RANKL plays an important role in the metabolism of a normal bone, but it is also deeply involved in the development of bone-related diseases. For example, in postmenopausal osteoporosis, the number of osteoclasts and their bone resorbing activity are increased, and the bone mass decreased. It is thought that RANKL is involved in the growth of osteoblasts [Kong, YY. et al., Nature 397:315-323 (1999)]. It has been demonstrated that a biological molecule called osteoclast formation inhibiting factor (to be referred to as "OPG/OCIF" herinafter) that interferes with the mutual binding of RANKL molecules not only promotes the osteoclast formation stimulated by RANKL, but also, in animal models having the diseases resulting from the enhanced osteoclast formation or enhanced activity of osteoclasts with bone-related diseases such as hypercalcemia, osteoporosis, etc., inhibits the osteoclast formation , and also improves the symptoms such as bone mass reduction and increase of calcium level in blood, by suppressing the bone absorbing activity. [Lacey DL., et al., Cell 93: 165-176 (1998)]. If it becomes possible to develop a method by which to inhibit the osteoclast formation stimulated by RANKL or induced via a certain agent stimulated by RANKL, as a

therapeutic means to recover the reduced bone mass or bone disruption in bone-related diseases resulting from the enhanced bone resorption such as osteopolosis, chronic rheumatoid arthritis, etc.; the reduced bone mass or the loosening after arthroplasty; or the disruption of a bone as a result of cancer metastasized to the bone, etc., the method will be prospective as a therapy to treat bone-related diseases.

The co-culture system used in this invention is a culture system in which osteoclast precursors and at least one type of cells which support osteoclast formation are cultivated in the same medium. The cell to support osteoclast formation is at least one cell selected from the group consisting of osteoblasts, stroma cells, fibroblasts, T-cells and B-cells. They may be used alone or in combination. Of those cells, stroma cells are preferably used, and bone marrow-derived stroma cells are more preferred. The most preferred combination consists of bone-marrow derived osteoclast precursors and bone marrow-derived stroma cells. The bone marrow-derived stroma cells may include mouse bone marrow-derived stroma cells such as ST-2, TSB-13-9, etc.

The cell line ST-2 is available from American Type Culture Collection, and it can be cultivated and maintained on the culture medium that will be detailed later, to be ready for experimentation.

The medium used in this invention to cultivate osteoclast precursors may include alpha MEM medium containing 8 to 1000 ng/ml of M-CSF and supplemented with 5 - 15% fetal bovine serum. Particularly, the alpha MEM medium containing 100 ng/ml of M-CSF and supplemented with 10% fetal bovine serum is preferred.

The medium used for the co-culture system may include alpha MEM medium supplemented with an induing factor described above, and 5 - 15% fetal bovine serum. Particularly, alpha MEM medium supplemented with 1 α,25-dihydroxy vitamin D3 having a concentration of 1 x 10^{-10}M to 1 x 10^{-7} M, and 10% fetal bovine serum is preferred. The inducing factor may be added to the medium during a period between a time when osteoclast precursors and osteoclast formation supporting cells such as bone marrow-derived stroma cells are inoculated concomitantly into the medium

and a time when osteroclasts are formed.

The ultrasound to be used in this invention includes ultrasound with an output too low to generate heat. More specifically, the ultrasound is a low-output pulsed ultrasound consisting of a series of bursts of which the wave component in a burst has a frequency of 1.3 to 2 MHz; the repetition frequency of bursts is 100 to 1000 KHz; the width of a burst is 10 to 2000 μsec; and the output intensity is 100 mW/cm^2 or less. Exposure of the ultrasound is achieved, to cite an example for illustration, as follows: an ultrasound generator provided by Exogen is so adjusted as to give low-output ultrasound pulses lasting 20 minutes in total for a day; the generator is so positioned with respect to the culture as to expose the wave to the bottom of the culture well; and the exposure continues for three to five days. The preferred properties of the ultrasound exposure, for example, are as follows; the width of a burst was 200 μsec, the frequency was 1.5 MHz, the repetition frequency of bursts was 1 kHz and the output intensity was 30 mW/cm^2.

The ultrasound generator to be used in this invention is not limited to any specific one, but, if clinically used, what is preferred is, for example, a generator enabling basic, non-invasive therapy as disclosed in US patent (No. 4,530,360) conferred to Duarte, which is capable of applying pulsed ultrasound percutaneously from a point close to the site to be treated. The preferred ultrasound emitted by such a generator may consist of a series of bursts of which the wave component in a burst has a frequency of 1.3 to 2 MHz; the repetition frequency of bursts is 100 to 1,000 Hz; the width of a burst is 10 to 2,000 μsec; and the output intensity is 100 mW/cm^2 or less. The duration of exposure is preferably shorter than 20 minutes for a day. Or, an ultrasound-based therapeutic system incorporating a body applicator unit communicable with a remote control unit as disclosed in US Patent (No. 5,003,965) conferred to Talish et al. may be used. The ultrasound in this case is a low-output pulsed ultrasound with a frequency of 1.3 to 2 MHz and an output intensity of 1 to 50 mW/cm^2.

According to the method of this invention for inhibiting the osteoclast formation or bone resorption, it will be possible to expose ultrasound onto a lesion where bone mass reduction or bone disruption occurs as a result of the

enhanced osteoclast formation or enhanced bone resorption, and, if it is clinically applied, to recover the reduced bone mass or bone disruption in bone-related diseases such as osteopolosis, chronic rheumatoid arthritis, etc., to fix aseptic loosening after arthroplasty, and to treat the bone disruption due to a tumor metastasized to a bone. Moreover, the method of this invention for the inhibiting osteoclast formation or bone resorption is comparatively free from the risk of causing side-effect as compared with the conventional therapy based on the administration of medicines.

Examples

The present invention will be described below by means of examples.

Examples 1-1 to 1-4, and Comparative Examples 1-1 to 1-4

Mouse bone marrow-derived osteoclast precursors (to be referred to as "MDBM" hereinafter) were obtained by extracting the bone marrow of femur and tibia of mice, separating monocytes according to convention, inoculating the monocytes on dishes to give a concentration of 4 x 10^6 cells/10 cm-dish, and selectively cultivating adhesive cells in the presence of 100 ng/ml of M-CSF for three days. The culture medium was an alpha MEM medium containing 100 ng/ml of M-CSF and supplemented with 10% fetal bovine serum . MDBM was inoculated into a 6-well plate 2 ml for each well to give a concentration of 1 x 10^5 cells/well. To each well, soluble RANKL (to be referred to as "sRANKL" hereinafter) was added to give a concentration of 50 ng/ml. sRANKL was obtained from Repro-Tech. For the experiment, following groups were prepared.

Comparative Example 1-1: control group cultivated for 72 hours.

Example 1-1: ultrasound exposure group cultivated for 72 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for two days successively.

Comparative Example 1-2: control group cultivated for 96 hours.

Example 1-2: ultrasound exposure group cultivated for 96 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for three days successively.

Comparative Example 1-3: control group cultivated for 108 hours.

Example 1-3: ultrasound exposure group cultivated for 108 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for four days successively.

Comparative Example 1-4: control group cultivated for 144 hours.

Example 1-4: ultrasound exposure group cultivated for 144 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for five days successively.

For both the Comparative Examples and Examples, sRANKL was dissolved in sterilized distilled water to give a concentration of 10 μg/ml; and the resulting solution was added so as to be diluted 200 times to give a concentration of 50 ng/ml. On the third day, the media of both the Comparative Examples and Examples were replaced with fresh 10% FBS-αMEM containing 50 ng/ml of sRANKL.

Exposure of ultrasound was not applied to the Comparative Examples 1-1 to 1-4. For Examples 1-1 to 1-3, a low output ultrasound was applied from the bottom of each well for 20 minutes daily using an ultrasound generating unit provided by Exogen which had been modified to be adapted for cell culture. Example 1-1 received ultrasound exposure for two days; Example 1-2 for three days; Example 1-3 for four days and Example 1-4 for five days successively. As to the output properties of the ultrasound applied, the width of a burst was 200 μsec, the frequency was 1.5 MHz, repetition frequency was 1 kHz and the output was 30 mW/cm^2.

Removal of the culture medium took place 72 hours after the onset of cultivation for Comparative Example 1-1 and Example 1-1, 96 hours after for Comparative Example 1-2 and Example 1-2, 108 hours after for Comparative Example 1-3 and Example 1-3, and 144 hours after for Comparative Example 1-4 and Example 1-4. The cells were fixed with phosphate-buffered formalin, stained with tartaric-acid-resistive acidic phosphatase (TRAP) according to convention for identification of osteoclasts, and placed under an optical microscope, and the number of osteoclasts was determined on the premise that a TRAP-positive polynucleated cell having ten or more nuclei is an osteoclast.

The results are shown in Figure 1. As is obvious from the Figure, the

numbers of osteoclasts in Examples 1-2 to 1-4 are less than those of Comparative Examples 1-2 to 1-4.

Examples 2-1 to 2-2 and Comparative Examples 2-1 to 2-2

MDBMs were obtained by extracting the bone marrow of femur and tibia of mice, separating monocytes therefrom according to convention, inoculating the monocytes on dishes to give a concentration of 4 x 10^6 cells/10 cm-dish, and selectively cultivating adhesive cells in the presence of 100 ng/ml of M-CSF for three days. The culture medium was an alpha MEM medium containing 100 ng/ml of M-CSF and supplemented with 10% fetal bovine serum . The cell line TSB-13-9 derived from mouse bone marrow-derived stroma cells is an established cell line prepared from cells obtained from the bone marrow of SV-40-large-T-antigen transgenic mice. The cells of TSB-13-9 were inoculated on petri dishes to give a concentration of 2 x 105 cells/10 cm-dish, and cultivated for three days. The medium was an alpha MEM medium supplemented with 10% fetal bovine serum.

Cells of MDBM and TSB-13-19 were simultaneously inoculated into a 6-well plate 2 ml for each well to give concentrations of 1 x 10^5 and 1 x 10^6 cells/well, respectively. To each well, 1 x 10^{-8} M 1α,25-dihydroxyvitamin D3 was added. For the experiment, following groups were prepared.

Comparative Example 2-1: control group cultivated for 96 hours.

Example 2-1: ultrasound exposure group cultivated for 96 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for three days successively.

Comparative Example 2-2: control group cultivated for 120 hours.

Example 2-2: ultrasound exposure group cultivated for 120 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for four days successively.

For both the Comparative Examples and Examples, 1α,25-dihydroxyvitamin D3 was dissolved in ethanol to give a concentration of 1 x 10^{-5}M; and the resulting solution was added so as to be diluted 1,000 times to give a concentration of 1 x 10^{-8} M. On the third day, the media of both the Comparative Examples and Examples were replaced with fresh 10%

FBS— α MEM containing 1 x 10^{-8} M 1 α ,25-dihydroxyvitamin D3.

Exposure of ultrasound was not applied to Comparative Examples 1-1 and 1-2. For Examples 1-1 and 1-2, low output ultrasound was applied from the bottom of each well for 20 minutes daily using an ultrasound generating unit provided by Exogen which had been modified to be adapted for cell culture. Example 1-1 received the ultrasound exposure for three days successively, and Example 1-2 for four days. The ultrasound consisted of the output properties, the width of a burst was 200 μsec, the frequency was 1.5 MHz, the repetition frequency of bursts was 1 kHz and the output intensity was 30 mW/cm^2.

Removal of the medium took place 96 hours after the onset of cultivation for Comparative Example 1-1 and Example 1-1, and 120 hours for Comparative Example 1-2 and Example 1-2. The cells were fixed with 10% phosphate-buffered formalin, stained with tartaric acid resistive, acid phosphatase (TRAP) according to convention for identification of osteoclasts, and placed under an optical microscope, and the number of osteoclasts was determined on the premise that a TRAP-positive polynucleated cell having ten or more nuclei is an osteoclast. The results are shown in Figure 1.

As is obvious from Figures 2 and 3, the numbers of osteoclasts in Examples 1-1 and 1-2 are less than those of Comparative Examples 1-1 and 1-2.

Examples 3-1 and Comparative Example 3-1

MDBMs prepared as in Example 1 were inoculated onto a 24-well plate 1 ml for each well to give a concentration of 2 x 10^4 cells/well. To each well of the 24-well plate, a hydroxyapatite-coated disc (Osteologic, Millennium Biologix, Inc.) had been placed in advance to receive the cells to grow them thereupon. sRANKL was added to each well to give a concentration of 50 ng/ml. sRANKL had been obtained from Pepro-Tech.

For the experiment, following groups were prepared.

Comparative Example 3-1: control group cultivated for 120 hours.

Example 3-1: ultrasound exposure group cultivated for 120 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for four days successively.

For both the Comparative Examples and Examples, sRANKL was dissolved in sterilized distilled water to give a concentration of 10 μg/ml; and the resulting solution was added so as to be diluted 200 times to give a concentration of 50 ng/ml. On the third day, the medium of both the Comparative Examples and Examples were replaced with fresh 10% FBS− α MEM containing 50 ng/ml of sRANKL.

Exposure of ultrasound was not applied to Comparative Example 3-1. For Example 3-1, a low output ultrasound was applied from the bottom of each well for 20 minutes daily for four days successively using a ultrasound generating unit provided by Exogen which had been modified to be adapted for cell culture. The ultrasound consisted of the output properties, the width of a burst was 200 μsec, the frequency was 1.5 MHz, the repetition frequency of bursts was 1 kHz and the output intensity was 30 mW/cm^2.

Removal of the medium took place 120 hours after the onset of cultivation for both Comparative Example 3-1 and Example 3-1, and the hydroxyapatite-coated discs were removed. The disc was washed with distilled water, and placed under an optical microscope, and absorption of the hydroxyapatite mass by osteoclasts was checked. A picture of a part of the disc (whose area is 50% or more of the total area) was fed through an image processor into a computer, and the area of disc undergoing resorption was determined by means of an image analysis application. The resorbed area relative to the total was determined and the results are shown in Figure 3.

As is obvious from Figure. 3, the relative area of disc subjected to osteoclastic resorption is smaller in Example 3-1 than in Comparative Example 3-1.

Example 4-1 and Comparative Example 4-1

MDBM and the cell line TSB-13-9 derived from mouse bone-marrow derived stroma cells were prepared as in Example 2. The medium was an alpha MEM medium supplemented with 10% fetal bovine serum .

MDBM and TSB-13-19 were inoculated at the same time into a 24-well plate to give concentrations of 2×10^4 and 1×10^5 cells/well, respectively. The volume of the culture medium was arranged as 1 ml finally. To each

well of the 24-well plate, a hydroxyapatite-coated disc (Osteologic, Millennium Biologix, Inc.) had been placed in advance to receive the cells to grow them thereupon. 1 α,25-dihydroxyvitamin D3 was added to each well to give a concentration of 1 x 10^{-8} M.

For the experiment, following groups were prepared.

Comparative Example 4-1: control group cultivated for 120 hours.

Example 4-1: ultrasound exposure group cultivated for 120 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for four days successively.

For both the Comparative Example and Example, 1α,25-dihydroxyvitamin D3 was dissolved in ethanol to give a concentration of 1 x 10^{-5}M; and the resulting solution was added so as to be diluted 1,000 times to give a concentration of 1 x 10^{-8} M. On the third day, the media of both the Comparative Example and Example were replaced with fresh 10% FBS— α MEM containing 1 x 10^{-8} M 1 α,25-dihydroxyvitamin D3.

Exposure of ultrasound was not applied to Comparative Example 4-1. For Example 4-1, low output ultrasound was applied from the bottom of each well for 20 minutes daily for four days successively using an ultrasound generating unit provided by Exogen which had been modified to be adapted for cell culture. The ultrasound consisted of the output properties, the width of a burst was 200 μsec, the frequency was 1.5 MHz, the repetition frequency of bursts was 1 kHz and the output intensity was 30 mW/cm^2.

Removal of the medium took place 120 hours after the onset of cultivation for both Comparative Example 4-1 and Example 4-1. The disc was washed with distilled water, and placed under an optical microscope, and resorption of the hydroxyapatite mass by osteoclasts was checked. A picture of a part of the disc (whose area is 50% or more of the total area) was fed through an image processor into a computer, and the area of disc undergoing absorption was determined by means of an image analysis application. The resorbing area relative to the total was determined and the results are shown in Figure 4.

As is obvious from Figure 4, the relative area of disc subjected to osteoclastic resorption is smaller in Example 4-1 than in Comparative

Example 4-1.

The present invention provides a method for inhibiting osteoclast formation or a method for inhibiting bone resorption, characterized in using the exposure of ultrasound. A clinical application to the medical treatment for bone-related deceases, such as a bone mass reduction or a bone disruption by osteopolosis and chronic rheumatoid arthritis, a bone destruction of aseotic loosening with joint arthroplasty just after the replacement with an artificial joint, by ultrasound exposure, which is physical and non-invasive, to a lesion where bone mass reduction or bone destruction occurs as a result of the enhanced osteoclast formation or enhanced bone resorption, without some side-effects, becomes possible. Moreover, the clinical application of the method of this invention for inhibiting the osteoclast formation or bone resorption will be promising in future, because the risk of causing side-effect is smaller in this method as compared with the conventional therapy based on the administration of medicines.